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INFLUENCE OF EXCESS VITAMIN A ON THE SULPHATE METABOLISM OF BONE RUDIMENTS GROWN *IN VITRO*

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In organ culture experiments, Fell & Mellanby (1952) found that when vitamin A was added to the culture medium (+A medium) of long-bone rudiments from chick and mouse embryos, the intercellular material of the cartilage rapidly lost its capacity for metachromatic staining, shrank and finally disintegrated; the chondroblasts usually appeared healthy throughout this process. Later, the effect of excess vitamin A on the differentiation *in vitro* of the early embryonic epidermis of the chick was investigated (Fell & Mellanby, 1953); in normal medium the ectoderm formed squamous, keratinizing epithelium, but in +A medium keratinization was suppressed and the epithelium developed into a mucus-secreting, often ciliated membrane resembling the normal nasal mucosa.

The transformation of the epidermis into a mucous instead of a keratinizing epithelium was accompanied by a profound alteration in the sulphate metabolism (Fell, Mellanby & Pelc, 1954). This was shown by treating ectodermal explants, grown in +A and in normal medium respectively, with labelled sodium sulphate ($Na_2{}^{35}SO_4$) and studying the distribution of the isotope by means of autoradiographs (ARG). In the present investigation similar experiments were made to elucidate the sulphate metabolism of long-bone rudiments grown in normal and in +A medium. For comparison, the *in vivo* uptake of sulphate in the long bones of chick embryos was studied.

MATERIALS AND METHODS

Femora and tibiae from 6-day chick embryos were grown by the watch-glass method (Fell & Robison, 1929; Fell & Mellanby, 1952) in a mixture of 3 parts of fowl plasma and 1 part of chick embryo extract. This mixture was supplemented with approximately 0·2% of glucose. Normal medium contained 250–300 i.u. of vitamin A/100 ml., and +A medium 2800–3000 i.u./100 ml. The rudiments were transferred to fresh medium every 2 days. Labelled sulphate was obtained

from the Radiochemical Centre, Amersham, England. One drop of Tyrode solution containing 200μc $Na_2{}^{35}SO_4$/ml. was deposited on each explant and the remaining liquid pipetted off after 30 sec.

The explants were fixed in acetic acid–alcohol (1:3; 30 min) followed by formol saline (60 min), embedded in paraffin wax and serially sectioned. This method of fixation has been used successfully in previous investigations and has the advantage of combining good preservation of the cells with freedom from artifacts in autoradiography. It has the disadvantage that flattening does not eliminate folds from sections of cartilage fixed in this way. Autoradiographs of the sections were prepared by the stripping film technique (Doniach & Pelc, 1950). One slide of each bone was exposed for 7–9 days and, after photographic processing, stained with toluidine blue. The slide was then treated with 10% acetic alcohol, which largely decolorized the photographic film but not the underlying sections; after being washed in tap water, it was passed through the increasing concentrations of ethanol to xylene and mounted in DPX. The second slide was exposed for 19–25 days and left unstained; the ARG was sufficiently heavy for observation with a pair of projection microscopes, which facilitated comparison of sections.

Expt. in vivo. Eggs were injected with 200μc $Na_2{}^{35}SO_4$ per egg after 7, 9 or 13 days' incubation, opened after 4 or 24 hr, and ARG's of the long bones prepared.

Expt. no. 1. After 4 days' growth in normal or +A medium, explanted bones were treated with $Na_2{}^{35}SO_4$ and fixed 2, 4, 24 or 48 hr later.

Expt. no. 2. After 4 days in normal or +A medium, $Na_2{}^{35}SO_4$ was applied; 24 hr later the explants were transferred to non-radioactive medium for 4, 24 or 48 hr and fixed.

Expt. no. 3. After 2, 4, 6, 8 or 10 days in normal or +A medium, $Na_2{}^{35}SO_4$ was applied; 4 hr later some of the explants were fixed, while others were washed in dilute embryo extract and transferred to non-radioactive medium for 24 or 48 hr and then fixed.

Expt. no. 4. Explants were grown for 4 or 6 days in normal medium, then for 4 days in +A medium; after this $Na_2{}^{35}SO_4$ was applied for 30 sec, then soaked off and 4 hr later one half of the bones were fixed. The other half were transferred to unlabelled + A medium and fixed 2 days later. For an equal number of explants the procedure was reversed, i.e. the explants were grown for 4 or 6 days in +A medium followed by 4 days in normal medium, then treated with $Na_2{}^{35}SO_4$ for 4 hr; half the number were fixed immediately, and the rest after 2 days in normal unlabelled medium.

Altogether 168 explants were used in the experiments.

RESULTS

Experiment in vivo. In bones fixed 4 hr after injection, the $^{35}SO_4$ is concentrated in the cells (Pl. 1, fig. 1): after 24 hr an appreciable concentration is found in the matrix as well (Pl. 1, fig. 2), confirming results of previous experiments on the renewal of chondroitin sulphate in the cartilage of adult mice (Pelc & Glücksmann, 1955). It can be concluded that in normal cartilage $^{35}SO_4$ is bound in an organic substance or substances first within the cartilage cells; this material, which is probably chondroitin sulphate, or a precursor which is not removed by fixation, etc., then passes into the matrix.

The bone rudiments from eggs injected after 7 days' incubation show almost uniform incorporation of $^{35}SO_4$ (Pl. 1, fig. 3) throughout the cartilage, which at this stage is still continuous in all the long bones, as the marrow cavity is not yet formed. The ARG is weaker above the hypertrophic cells in the middle portion of the shaft.

After 9 days' incubation the first signs of the marrow cavity have appeared in the middle of the shaft of the femur, humerus and tibia, but not in the ulna and radius. After 4 hr contact with $^{35}SO_4$ the uptake of the isotope is confined to the epiphyses, to the zones of flat, proliferating cells, young hypertrophic cells (cf. Amprino, 1954) and to a relatively narrow zone in the middle of the shaft (Pl. 1, fig. 4), in which the cells are more tightly packed than in the adjacent regions of mature hypertrophic cartilage which show no ARG. This narrow zone of ARG coincides with the part of the cartilage which is destined to disappear first when the marrow cavity begins to be excavated. The bones from eggs injected after 9 days' incubation and fixed 24 hr later have well-developed marrow cavities (Pl. 1, fig. 5). $^{35}SO_4$ is still found where it was at 4 hr but, as would be expected, the ARG is much heavier, since 24 hr instead of 4 hr were available for incorporation. The cells in the newly formed marrow cavity are weakly labelled; the periosteal bone is also labelled. The bones from embryos incubated for 13 days show essentially the same uptake as after 9 days; some cells in the marrow cavity give a strong ARG (cf. Lajtha, Ellis & Oliver, 1953).

Control explants in normal medium. Two and four hours after the application of the $^{35}SO_4$ (Expts 1, 3), in all rudiments the sulphate is concentrated more in the cells than in the matrix. In the 2-day explants (Expt. 3), which are at an early stage of differentiation, are still growing actively and are forming matrix throughout their length, the sulphate is taken up intensely by the whole rudiment, though uptake is greatest in the zones of flattened cells and in the epiphyses. In the 4-day (Pl. 2, fig. 6) and still more in the 6-day explants (Pl. 2, fig. 7), however, in which development is more advanced, the heaviest ARG is over the young hypertrophic cells in the distal parts of the shaft where matrix-formation is probably maximal; the ARG is also dense over the small-celled epiphyses and in the two proliferative zones of flattened cells. As hypertrophy increases, the uptake in the middle segment of the shaft, where chondrogenesis is rapidly coming to an end, becomes progressively less with age, although a small proportion of cells continue to bind $^{35}SO_4$ (Pl. 2, fig. 7); this diminution begins in the interior of the hypertrophic zone, i.e. in the oldest cartilage, and spreads to the surface. The young periosteum maintains synthesis, however, and a weak ARG is seen over the newly formed sheath of bone on the surface of the hypertrophic cartilage.

When the explants are transferred after 4 hr in labelled medium to non-radioactive medium for 24 hr (Expt. 3), the $^{35}SO_4$, though still plentiful in the cells, is more evenly diffused throughout the cartilage. The intensity of the ARG as seen by low power seems diminished in the epiphyses, but the total content of $^{35}SO_4$ is about the same in the young hypertrophic cells and in the middle segment of the shaft. To some extent it has merely become more dispersed as a result of the expansion of the growing rudiment. Incubation

on non-radioactive medium for a further 24 hr produces a still greater diffusion of the ARG, the matrix often containing much more $^{35}SO_4$ than the cells.

The rudiments grown continuously for 24–48 hr in labelled medium (Expt. 1) show the same trend, but results are less clear-cut because labelled sulphate is available for up to 2 days. For this reason the difference between cells and matrix is less pronounced. Similarly, explants grown in labelled medium for 24 hr and then transferred (Expt. 2) give the same result as those of Expt. 3, but the situation is more complicated because some sulphate is already concentrated in the matrix at the time of transfer to non-labelled medium.

+A explants. As in the controls, 4 hr after the application of the $^{35}SO_4$ more sulphate is present in the cells than in the matrix. Throughout the series, the femur is more affected by the +A than the tibia. After 2 days in +A medium, the matrix of the femur shows a slightly weakened metachromatic stain in the peripheral regions of the epiphyses and of the proliferative zones of flattened cells, but no histological change can be detected in the tibia. The regions of reduced metachromasia have taken up rather less sulphate than the rest of the femur and throughout both rudiments the uptake seems to be somewhat lower than in the corresponding controls. After 4 days' growth, the loss of metachromatic stain from the peripheral matrix of the zones of flattened cells is more pronounced in the femur and has now appeared in the tibia also; the uptake of $^{35}SO_4$ is greatly reduced in the matrix that fails to stain with toluidine blue, but elsewhere is only slightly less dense than in the controls (Pl. 2, fig. 8). In the 6-day +A explants, the zones of flattened cells show similar but more extensive areas of nearly unstained matrix with greatly diminished uptake, but in addition peripheral regions of the hypertrophic zone, though still staining with almost normal intensity, take up much less $^{35}SO_4$ than in the controls. In some rudiments at this stage the changes are more advanced; in the middle segment of the shaft the matrix has almost completely lost its affinity for toluidine blue and the ARG is very sparse.

Twenty-four hours after transfer to non-radioactive +A medium, the ARG in the +A explants, as in the controls, is less concentrated in the cells and more evenly distributed between the cells and matrix. The 2-day explants show a slight loss of metachromasia and less ARG at the periphery of the zones of flattened cells when examined 24 and 48 hr after transfer, but otherwise do not differ from the controls. A greater change appears in the 4-day explants when grown for a further 24–48 hr on non-radioactive +A medium. In the femur fixed 48 hr after transfer, the matrix of a large part of the zone of flattened cells has lost its affinity for toluidine blue, is much reduced in amount and has almost completely lost its ARG, although some remains in the cells; metachromasia and $^{35}SO_4$ have almost disappeared from the shaft. The tibiae fixed after

24 and 48 hr on non-radioactive +A medium show similar but usually less pronounced changes.

The femora cultivated for 6 days in +A medium are drastically affected after further growth on non-radioactive +A medium. Twenty-four hours after transfer there is much peripheral loss of metachromasia from the zones of flattened and young hypertrophic cells, and while some ^{35}S remains in the cells, it has almost completely gone from the intercellular material; other parts of the rudiment also show such peripheral changes, though to a lesser degree.

TABLE 1. (Expt. 3.) Comparison of autoradiograph and metachromatic staining of the shafts of bones grown in +A and in normal medium

		Normal medium						+A medium					
Days before SO_4		2, 4	4	4	6	6	6	2, 4	4	4	6	6	6
Days after SO_4		0	1	2	0	1	2	0	1	2	0	1	2
Femora	M	4	4	4	4	4	4	4	4	3	4	1	0
	ARG	4	3	3	3	4	3	4	3	0	0	0	0
Tibiae	M	4	4	4	4	4	4	4	4	4	4	3	3
	ARG	4	3	4	3	4	3	4	3	3	3	0	0

M, metachromatic stain: 0, none; 1, slight; 3, strong; 4, very strong.
ARG: 0, none; 3, strong; 4, very strong.

After 48 hr (Pl. 2, fig. 9), the matrix has almost disappeared from some zones of flattened cells, and the $^{35}SO_4$ is greatly reduced in both cells and matrix; the peripheral matrix of the epiphyses has lost its characteristic staining and most of its $^{35}SO_4$, but the interior still stains normally and has an intense ARG. There is no uptake of $^{35}SO_4$ in the mature hypertrophic cells of the shaft, even in regions which still show metachromasia. Relative values for the intensity of metachromatic staining and ARG in the shaft are given in Table 1 (Expt. 3). In tibiae cultivated for 6 days the changes are similar but less advanced.

A time-lag between the disappearance of $^{35}SO_4$ and of metachromasia can be seen in Table 1. Metachromatic staining in the shaft of the femora is intense up to the 6th day of cultivation in +A medium and then diminishes rapidly. All the $^{35}SO_4$ which was taken up in the shafts of the 4-day explants has disappeared 2 days later, and subsequently the cells cease to utilize $^{35}SO_4$. A similar effect appears about 2 days later in the tibiae. The connexion between intensity of metachromatic staining and concentration of chondroitin sulphate is not known; and further, since staining was not standardized in our experiments and our estimate of the intensity of the stain is subjective, we cannot state what loss of chondroitin sulphate is equivalent to a given change in the staining reaction. It can be assumed, however, that a drop from our grade 4 to grade 0 represents almost complete disappearance of chondroitin sulphate. For the ARG's a quantitative estimate of the change is easier, and a drop from grade 4 to grade 0 is equivalent to a loss of 95% or more of the isotope. It can

be concluded that the time-lag between the disappearance of $^{35}SO_4$ and of metachromasia is a real difference.

Mixed treatment (*Expt.* 4). Pl. 2, figs. 10–12, show stained sections and ARG of explants which were cultivated either first in normal medium and then in +A medium, or first in +A and then in normal medium. It is seen that rudiments grown first in +A medium are much more affected than those which were first cultivated on normal medium. For example, the rudiment shown in Pl. 2, fig. 10 (4 days normal + 4 days +A) is only bent, and the centre of the shaft is well stained, whilst that in Pl. 2, fig. 12 (4 days +A +4 days normal) is broken and distorted. Two days later (Pl. 2, fig. 11, 4 days normal + 6 days +A) the middle of the shaft is severely affected and bent sharply. These rudiments grown first in normal medium thus differ in an important respect from those continuously grown in +A medium, which show a constriction in the zone of proliferating cells before the shaft is drastically altered (Fell & Mellanby, 1952). Conversely, the region of proliferating cells is much less affected than in rudiments grown only in +A medium. The sequence of damage in rudiments first grown in +A medium and then in normal seems to follow the pattern of continuous growth in +A medium. All the explants are severely damaged; the epiphysis has been detached from the shaft, the remains of which are relatively straight. As far as the experiments overlap, these results agree with Herbertson's findings (1955).

DISCUSSION

The uptake of $^{35}SO_4$ by the control explants in normal medium is very similar to that of the normal long-bone rudiments in the embryo. In both, the sulphate is first concentrated in the cells and then appears in the matrix; the ARG is most intense over the epiphyses, the proliferative regions and the newly formed distal hypertrophic cartilage; as development proceeds it becomes progressively less in the old hypertrophic cartilage in the middle of the shaft, indicating that the synthesis of matrix is diminishing in this region. The main difference between the normal long bone in the chick and the control explants is the presence in the former of a narrow zone of ARG over an area of closely packed cartilage cells in the middle of the shaft (Pl. 1, fig. 4*b*). This region is the first to be eroded when the narrow cavity is formed, and it is absent in the control explants *in vitro* in which no marrow cavity develops; this uptake of $^{35}SO_4$ suggests that the area may be the site of catabolic processes preceding dissolution.

When the control explants are compared with those grown in +A medium the following facts emerge: (1) in explants grown in normal medium, the cartilage of the shaft retains all or most of the $^{35}SO_4$ once it has been incorporated; (2) in the explants grown for 4 days in +A medium, $^{35}SO_4$ is taken up, but after a further 2 days in unlabelled +A medium much of the sulphate is lost, especially from the middle of the shaft and the periphery; (3) after about

6 days' growth in +A medium, the peripheral cartilage and the middle segment of the diaphysis cease to incorporate $^{35}SO_4$; (4) explants grown first in +A and then in normal medium are more severely damaged than those grown first in normal and then in +A medium.

Our experiments indicate, therefore, that vitamin A both inhibits the synthesis of new cartilage matrix and causes the dissolution of that already formed. These results could be explained, at least in part, by the following hypothesis: (*a*) excess vitamin A causes cells either to elaborate a new enzyme which converts chondroitin sulphate to a more soluble form, or to produce excessive amounts of an enzyme which is already present in the tissue: (*b*) this enzyme diffuses into the matrix, and (*c*) once cells have begun to produce this enzyme, they continue to do so. Chondroitin sulphate in normal adult cartilage is continuously broken down in the matrix and replenished by the cells (Pelc & Glücksmann, 1955). It is therefore likely that an enzyme which hydrolyses chondroitin sulphate is normally present.

Under the influence of excess vitamin A the concentration of this hypothetical enzyme will at first be high in the cells, but when sufficient quantities have been secreted into the matrix the existing chondroitin sulphate will be affected. There will therefore be a time-lag between the production of this soluble sulphated compound and the dissolution of old matrix. This would account for the time-lag between the disappearance of bound $^{35}SO_4$ and that of metachromatic staining (see Table 1). It is hoped to test this hypothesis by experiments on the uptake of ^{14}C-labelled vitamin A and by comparing the enzymic action of extracts of vitamin-A-treated and untreated explants.

As described above, the effect of vitamin A is not uniform throughout the rudiment. Thus the loss of metachromatic staining and of sulphate uptake begins near the surface and spreads inwards; this may be correlated with the inward diffusion of the vitamin from the culture medium. In addition, the old hypertrophic cartilage of the shaft is more rapidly and severely affected than the distal cartilage. It has been shown that normally the synthesis of matrix is waning in the middle of the shaft while it is still active distally; it might be expected that this natural decline in the synthetic processes would be hastened by the vitamin, so that matrix formation would be arrested in this region before it ceased in other parts of the rudiment.

Dziewiatkowsky (1954) has investigated the effect of vitamin A on the sulphate metabolism of vitamin-A-deficient rats. He found that the administration of vitamin A causes a higher uptake of sulphate in the mucopolysaccharide of the end of the femur and in the skin after a delay of about 48 hr. This increased uptake (Dziewiatkowsky, 1954, text-figs. 1, 2) is probably due to unbound sulphate since it does not appear in the series where the unbound sulphate has been removed (Dziewiatkowsky, 1954, text-fig. 4). This author's autoradiographs also indicate a quicker release of bound sulphate from the epiphysial

cartilage after vitamin A was given to deficient animals. Dziewiatkowsky's results are thus comparable to ours, which show very little effect of excess vitamin A on explanted limb-bone rudiments during the first 2 days in +A medium, but a rapid disappearance of the ^{35}S after more prolonged exposure to the vitamin.

SUMMARY

1. The influence of excess vitamin A, on the sulphate metabolism ($Na_2{}^{35}SO_4$) of chick long-bone rudiments grown *in vitro* by the watch-glass method, was investigated by means of autoradiography.

2. The normal medium used for the controls (3 parts fowl plasma +1 part chick embryo extract+0·2% added glucose) contained 250–300 i.u. of vitamin A/100 ml., and the +A medium 2800–3000 i.u./100 ml. One drop of Tyrode solution containing 200μc$Na_2{}^{35}SO_4$/ml. was deposited on each explant and was pipetted off after 30 sec.

3. Some explants were fixed after 2, 4, 24 or 48 hours in labelled medium; others were transferred after 4 hours to unlabelled medium and fixed 24 or 48 hours later.

4. For comparison, eggs were injected with 200μc $Na_2{}^{35}SO_4$ after 7, 9 and 13 days' incubation, the long bones were fixed 4 or 24 hours later and serial sections were cut from which autoradiographs were made.

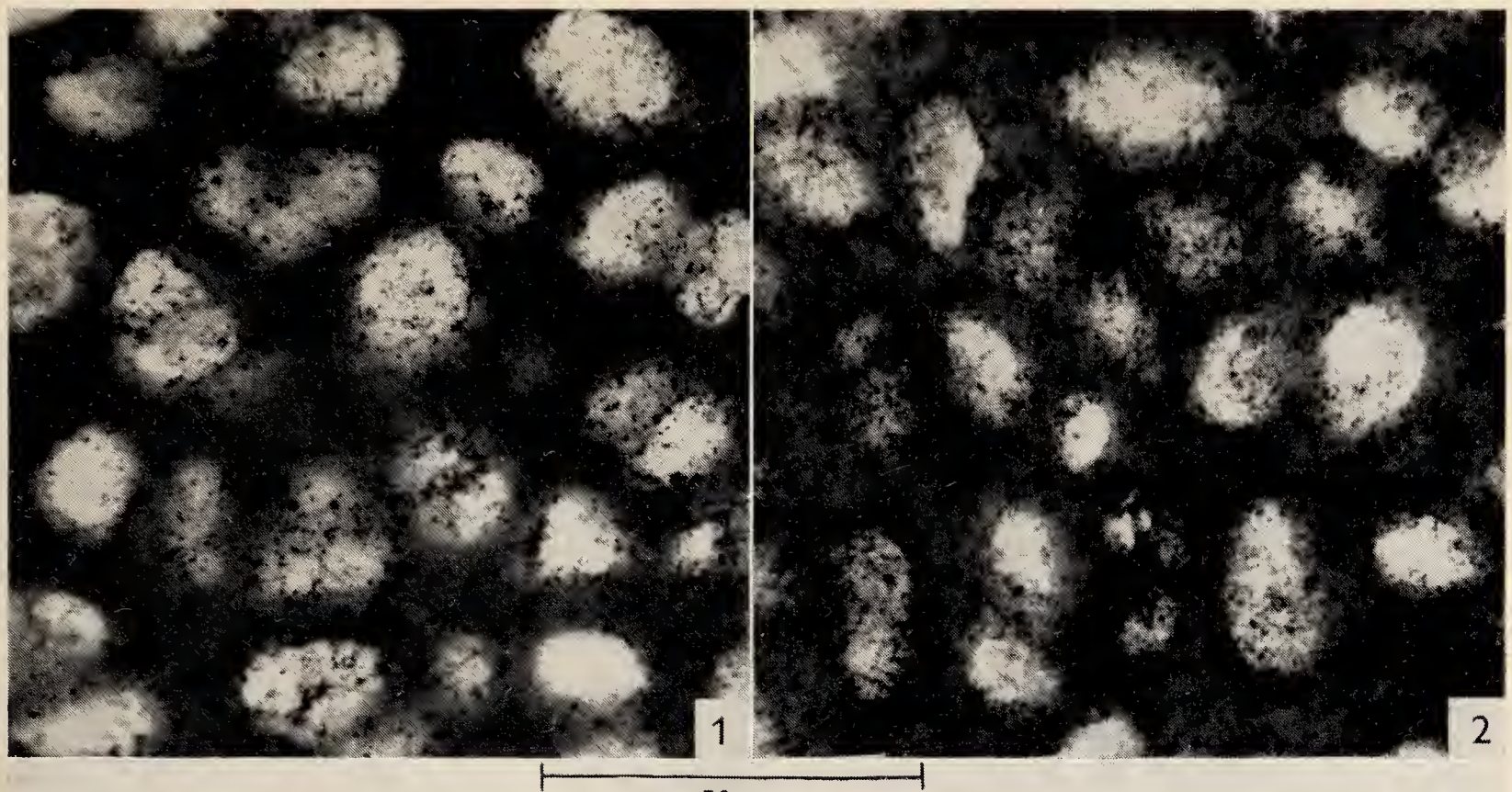
5. In all the bones, both normal and explanted, fixed 2 and 4 hours after labelling, sulphate was mainly concentrated in the cells; later, considerable quantities were found in the matrix, confirming earlier results on the sulphate metabolism of cartilage in mice.

6. In the rudiments from normal embryos, uptake was almost uniform throughout the cartilage in 7-day chicks; after 9 days' incubation it was confined to the epiphyses, the proliferative zones and a narrow region in the middle of the shaft; in 13-day rudiments the narrow zone of uptake in the middle of the shaft had been replaced by the invading marrow.

7. Control explants grown in normal medium showed essentially the same uptake as the embryonic rudiments labelled *in ovo*; when the control explants were transferred from labelled to unlabelled medium for 2 days little bound sulphate was lost.

8. In +A medium uptake diminished in the peripheral regions of the epiphyses and of the shaft. Two days after transfer to unlabelled medium there was an appreciable loss in the periphery of the shaft; this preceded the disappearance of metachromasia from the matrix, after which the cells were unable to bind sulphate.

9. If explants were first grown for 4–6 days in +A medium and then transplanted to normal medium for 4 days, the changes were similar to those produced by continuous culture in +A medium, but if normal medium were followed by +A medium, the effect was much less drastic.



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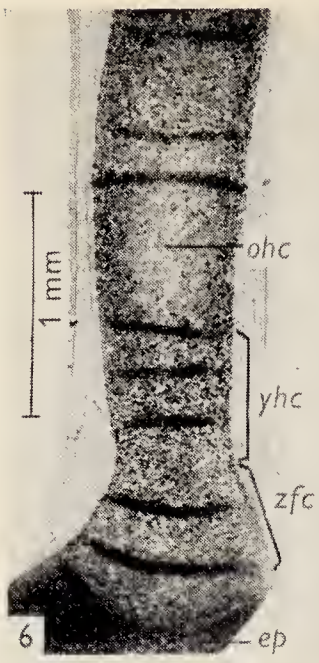

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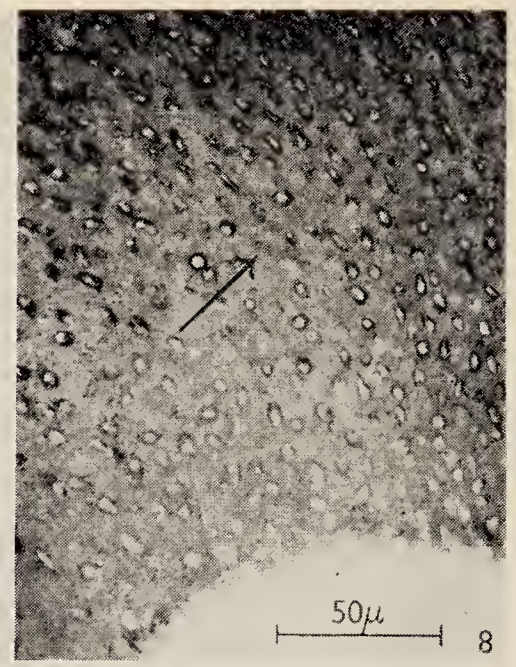

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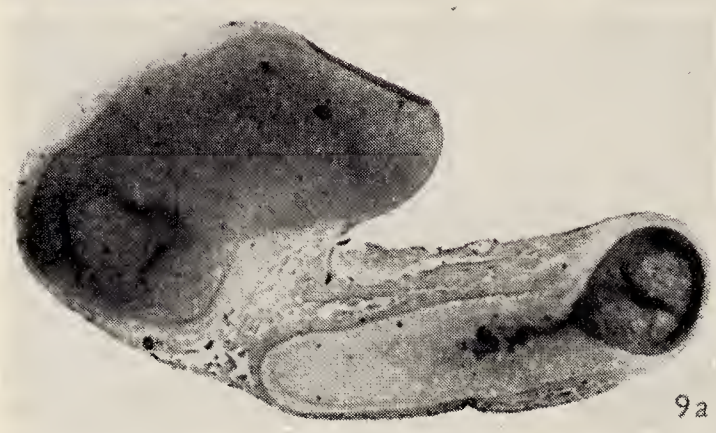
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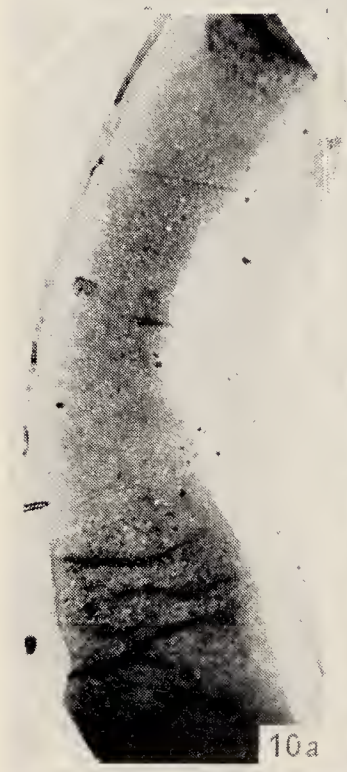
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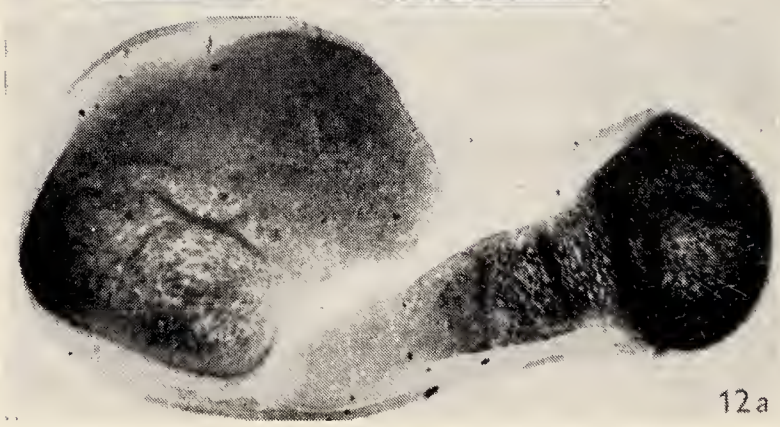
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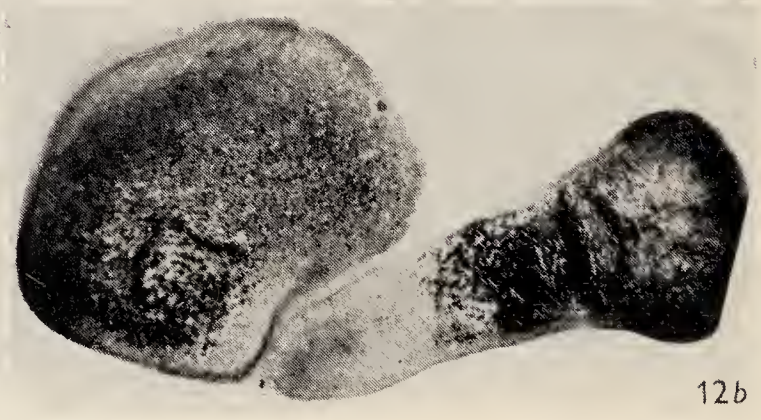
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10. It is tentatively suggested that, under the influence of vitamin A, the cartilage cells may produce an enzyme which results in the formation of a soluble sulphated mucopolysaccharide, instead of the normal chondroitin sulphate, and in the dissolution of existing matrix.

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EXPLANATION OF PLATES

ep	epiphysis	*ohc*	old hypertrophic cartilage
hc	hypertrophic cartilage	*yhc*	young hypertrophic cartilage
mc	marrow cavity	*zfc*	zone of flattened cells

Plate 1

Photographs of limb bones of normal embryos, from eggs into which $Na_2{}^{35}SO_4$ had been injected (*in vivo* experiment). Figures 3–5 are all to the same magnification.

Fig. 1. Hypertrophic cartilage from the femur of a 9-day embryo, fixed 4 hr after injection of $Na_2{}^{35}SO_4$. The ARG is mainly concentrated above the cells; slide exposed for 7 days, stained with toluidine blue.

Fig. 2. Hypertrophic cartilage from the femur of a 9-day embryo, fixed 24 hr after injection of $Na_2{}^{35}SO_4$. The ARG is now profuse over the matrix as well as over the cells; slide exposed for 7 days, stained with toluidine blue.

Fig. 3. Humerus from a 7 day embryo, 4 hr after injection of $Na_2{}^{35}SO_4$. The ARG is weak above the hypertrophic cells in the centre of the shaft; elsewhere the incorporation of the $^{35}SO_4$ is fairly uniform. (*a*), slide exposed for 7 days, stained with toluidine blue; (*b*) 21 days' exposure, unstained.

Fig. 4. Humerus from a 9-day embryo, 4 hr after injection of $Na_2{}^{35}SO_4$. The uptake of $^{35}SO_4$ is now confined to the distal parts of the bone, except for a narrow zone of ARG (marked with an arrow) in the middle of the shaft; (*a*) and (*b*) as for fig. 3.

Fig. 5. Humerus from a 10-day embryo, 24 hr after injection of $Na_2{}^{35}SO_4$. Excavation of the marrow cavity has begun in the middle of the shaft. (*a*) and (*b*) as before; the dark appearance in (*a*) is partly due to the heavy autoradiograph.

PLATE 2

Photographs of explanted femora and tibiae (Expt. 1, figs. 6–9; Expt. 4, figs. 10–12). Figs. 6, 7, 9 and 10–12 are to the same magnification.

Fig. 6. Tibia; 4 days in normal medium; $Na_2{}^{35}SO_4$, 4 hr. The ARG is heaviest over the young (distal and peripheral) hypertrophic cartilage and weakest over the oldest hypertrophic cartilage in the interior of the shaft; it is also dense over the epiphysis and zone of flattened cells. Slide exposed 19 days, unstained.

Fig. 7. Femur; 6 days in normal medium; $Na_2{}^{35}SO_4$, 4 hr. The ARG is very sparse over the old hypertrophic cartilage. (*a*) Slide exposed 7 days, stained with toluidine blue; (*b*) 19 days' exposure, unstained.

Fig. 8. Tibia; 4 days in +A medium; $Na_2{}^{35}SO_4$, 4 hr. Peripheral area in the zone of flattened cells; both the ARG and the coloration of the matrix progressively diminish towards the surface of the cartilage; the arrow indicates the long axis of the rudiment. 7 days' exposure, stained with toluidine blue.

Fig. 9. Femur; 6 days in +A medium; $Na_2{}^{35}SO_4$, 4 hr. The rudiment is greatly distorted, the peripheral matrix of the epiphysis has begun to lose its staining capacity and shows a diminished ARG, while, except in the interior, the shaft has lost both its affinity for toluidine blue and its capacity to take up $^{35}SO_4$ (*a*) and (*b*) as fig. 7.

Fig. 10. Femur; 6 days in normal medium + 4 days in +A medium; $Na_2{}^{35}SO_4$, 4 hr. The matrix in the middle segment of the shaft has not lost all its staining capacity, and the rudiment has preserved a fairly normal shape. (*a*) and (*b*) as fig. 7.

Fig. 11. Femur; 6 days in normal medium + 6 days in +A medium; $Na_2{}^{35}SO_4$, 4 hr. The shaft is more severely affected than in the explant shown in fig. 10. (*a*) and (*b*) as in fig. 7.

Fig. 12. Femur; 4 days in +A medium + 4 days in normal medium; $Na_2{}^{35}SO_4$, 4 hr. The explant is much more drastically altered than those grown first in normal and then in +A medium (figs. 10, 11). (*a*) and (*b*) as in fig. 7.